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This report describes the status of work under contract DAMD17-86-C-6150 at the end of the reporting period, March 15, 1989 to March 14, 1990. Analytical methodologies were under development for the determination of the concentrations of the anti-leishmanial compound, WR 6026 in blood and plasma; the radioprotective drugs WR 2721 and WR 3689 in plasma; the anti-malarial compound WR 238,605 in blood and plasma; the anti-malarial drug, mefloquine in plasma; the anti-viral drug, ribavirin, and WR 249,992 in plasma; the anti-malarial drug, ß-arteether, and dihydroginghaosu in biological fluids, the anti-malarial drug, halofantrine, and its metabolite, WR 178,460, in blood and plasma; the 4-hydroxymethyl and mono desethyl metabolites of WR 6026 (WR 254,421 and WR 211,789) in biological fluids; and the carboxyl metabolite of mefloquine, WR 160,972, in plasma. A study report was submitted for the WR 238,605 method. Routine assays for eight studies were underway during the fourth year of the contract for WR 6026 in human blood; WR 238,605 in beagle dog plasma and blood; pyridostigmine in human plasma; physostigmine in rat plasma, bile and tube bindings; and halofantrine in human plasma and blood. Analysis reports were submitted for eight studies.

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ANALYSIS OF INVESTIGATIONAL DRUGS IN BIOLOGICAL FLUIDS METHOD DEVELOPMENT AND ROUTINE ASSAY

Annual Report

May 15, 1990

Emil T. Lin, Leslie Z. Benet, Robert A. Upton and Winnie L. Gee

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6150

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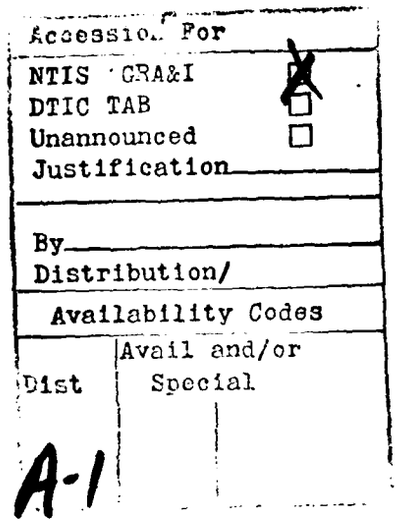
FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.



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INTRODUCTION

The laboratory has contracted (contract number DAMD17-86-C-6150) with the US military establishment to develop and use analytical methods for detection and quantitation of candidate chemical warfare antidotes, radioprotectants and anti-infectious disease drugs to support pharmacokinetic and bioavailability studies for the purpose of new drug development. The laboratory during the reporting period March 15, 1989 to March 14, 1990 completed development of one methodology for one drug (WR 238,605 in plasma and blood), began development of five methodologies (ribavirin and WR 249,992 in plasma and blood; ß-arteether and dihydroginghaosu in biological fluids; halofantrine and WR 178,460 in plasma and blood; 4-hydroxy metabolite of WR 6026 in biological fluids; and WR 160,972 (a mefloquine metabolite) in biological fluids) and ran routine analyses on 1957 specimens in support of 7 studies.

Reporting instructions required the completion of four quarterly project status and funds expenditure reports, one annual report, one study report, and eight routine analysis reports. In addition, revisions of selected reports from this contract were required. Previous related submittals were issued for contract DAMD17-86-C-6150, as detailed in Table 1.

No great difficulties are foreseen in completing methods development or performing routine analyses for the drugs and studies currently targeted. Increases in the workload past current levels, however, would require the hiring of additional personnel. The technical approach that is being used in the development process is to adapt high-performance liquid chromatographic methods to the analysis of specimens for each of the compounds of interest.

DISCUSSION

The technical work performed during the fourth year of the contract involved using accepted scientific procedures combined with the equipment and facilities of the University to obtain the data and to proceed with the development of methodologies as detailed elsewhere in this report. Accepted scientific procedures including normal and reversed phase high-performance liquid chromatographic methods, post column derivatization, and protein precipitation, extraction, and cartridge elution sample clean up procedures were employed in development and routine work. Tables 2 and 3 list the five chromatographic systems committed to development of methodologies, each complete with a Waters Intelligent Sample Processor (WISP) and a Hewlett Packard Integrator 3392A, and the nine chromatographic systems committed to the analysis of submitted specimens.

In addition, the development laboratory is equipped with three general purpose fume hoods, clean room facilities, three lockable explosion proof refrigerators, three -20°C and two -80°C freezers, one drug cabinet, two fire proof solvent cabinets and a

TABLE 1: PREVIOUS RELATED SUBMITTALS

Report Type	Number	Drug and Specimen Type	Date of Report
Annual	1		Apr. 13, 87
Annual (Revised)	1		Jun. 11, 87
Annual	2		Apr. 12, 88
Annual (Revised)	2		May 20, 88
Project Status	1		Jun. 30, 86
Project Status	2		Sep. 30, 86
Project Status	3		Jan. 27, 87
Project Status	4		Mar. 31, 87
Project Status	5		Jun. 26, 87
Project Status	6		Sep. 30, 87
Project Status	7		Dec. 23, 87
Project Status	8		Mar. 30, 88
Project Status	9		Jun. 30, 88
Project Status	10		Oct. 4, 88
Project Status	11		Jan. 4, 89
Project Status	12		Mar. 30, 89
Study	6	Mefloquine in plasma	May 4, 87
Study	6B		Oct. 19, 87
Study	6C		Jan. 8, 88
Study	7	Pyridostigmine in urine	May 12, 87
Study	7B		Jan. 12, 88
Study	8	Physostigmine in plasma	Oct. 20, 87
Study	8B		July 28, 88
Study	8C		Sept. 23, 88
Study	9	Physostigmine in plasma	May 27, 88
Study	9B		Sept. 12, 88
Study	10	WR 6026 in plasma and blood	Jan. 20, 89
Study	11	WR 2721 in plasma	Dec. 29, 88
Study	12	WR 3689 in plasma	Dec. 29, 88
Study	14	Mefloquine in plasma	Mar 13, 89

TABLE 1: PREVIOUS RELATED SUBMITTALS (CONTINUED)

Report Type	Report Number	Drug and Specimen Type	Date of Report
Analysis	AY86-1A	Halofantrine in plasma	Sep. 29, 86
Analysis	AY86-1B		Jun. 11, 87
Analysis	AY86-1C		Aug. 13, 87
Analysis	AY86-1D		Oct. 23, 87
Analysis	AY86-2A	WR 6026 in plasma	Oct. 29, 86
Analysis	AY86-2B		Feb. 12, 87
Analysis	AY86-2C		Apr. 23, 87
Analysis	AY86-2D		Jun. 24, 87
Analysis	AY86-3	Pyridostigmine in urine	Jan 27, 87
Analysis	Pyr/U 86-3B (revision of AY86-3)		Feb. 3, 88
Analysis	Mef/P 87-1	Mefloquine in plasma	Sep. 16, 87
Analysis	Mef/P 87-1B		Feb. 25, 88
Analysis	Pyr/PU 87-2	Pyridostigmine in plasma and urine	Jan 27, 88
Analysis	Pyr/PU 87-2B		Feb. 24, 88
Analysis	Pyr/P 88-1	Pyridostigmine in beagle dog plasma	Mar. 29, 88
Analysis	Pyr/P 88-1B		Apr. 26, 1988
Analysis	Pyr/P 88-2	Pyridostigmine in plasma	May 5, 1988
Analysis	Pyr/P 88-2B		June 13, 1988
Analysis	Pyr/P 88-2C		Aug. 3, 1988
Analysis	Pyr/P 88-3	Pyridostigmine in plasma	Aug. 2, 1988
Analysis	Phy/P 88-5	Physostigmine in beagle dog plasma	Aug. 26, 1988
Analysis	Phy/P 88-5B		Oct. 19, 1988
Analysis	Phy/P 88-6	Physostigmine in rhesus plasma	Sept. 15, 1988
Analysis	Phy/P 88-6B		Oct. 19, 1988
Analysis	Mef/P 88-11	Mefloquine in plasma	Dec. 8, 1988

60 sq. ft. solvent room. The routine analysis laboratory is equipped with two fume hoods, two explosion proof refrigerators, two -20°C freezers, one -80°C freezer, and one fire proof solvent cabinet These and all the usual laboratory equipment (e.g.,

balances, pH meters, incubators, centrifuges, pipets, etc.) necessary for preparing biological samples have been used in methods development and routine assays. During the first year of the contract, two refrigerated WISPs for the WR 2721 and WR 3689 assays, two Kratos post column reactors for the physostigmine assay, and two refrigerators for the South San Francisco laboratory were purchased. During the second year of the contract, two additional refrigeration units for WISP systems were purchased. During the third year of the contract, two RF-535 Shimadzu fluorescence detectors were purchased. During the fourth year of the contract, a biohazard hood and a -80°C freezer were purchased. In addition, since the first year of the contract, a Varian model 5000 gradient pump, two Varian model 8500 pumps, a Perkin Elmer 203 fluorescent detector, and two Shimadzu RF-530 fluorescent detectors were replaced with an LDC model CM 4000 gradient pump, two Rainin HPLX pumps, an Hitachi model 1000 fluorescent detector, and two Shimadzu RF-535 fluorescent detectors, respectively. An ESA electrochemical detector, the two Kratos reactors and a GC-MS were purchased with funds unrelated to the DAMD17-86-C-6150 contract, but this equipment can be made available, if needed, with DAMD17-86-C-6150 contract funding.

The facilities dedicated for use under this contract encompass rooms 822 and 824 of the Medical Sciences Building and rooms 1257 and 1258 of the Health Science East Building located at the University of California, San Francisco and the off campus laboratory at 296 Lawrence Dr., South San Francisco. The facilities occupy 3900 sq. ft. of space. Additional facilities at the South San Francisco laboratory will be available during the fifth year of the contract.

Data for an analysis is obtained by comparison of the results for a sample with the results for a series of standard curve samples. The standard curve is constructed by finding the best fit straight line with linear regression analysis of the peak height ratio of the drug to an internal standard versus the spiked concentration of prepared

TABLE 2: CHROMATOGRAPHIC SYSTEMS COMMITTED TO DEVELOPMENT

System	Pump	Detector
1	LDC CM 4000 gradient pump	Perkin Elmer 650-10S fluorescent detector
2	Beckman 110A	Perkin Elmer 204-A fluorescent detector
3	Waters 6000	Kratos Spectroflow 773 variable wavelength UV detector
4	Waters 6000	Kratos Spectroflow 773 variable wavelength UV detector
5	Beckman 100	Bioanalytical LC-4B electrochemical detector

TABLE 3: CHROMATOGRAPHIC SYSTEMS COMMITTED TO ROUTINE ANALYSIS

System	Pump	Detector	Attachment
1	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
2	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
3	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
4	Perkin Elmer series 3	Perkin Elmer 65 T variable wavelength UV detector with temperature controlled oven	WISP 710B and Integrator
5	Beckman 110B	Perkin Elmer 650-S fluorescent detector	WISP 710B and Integrator
6	Beckman 110B	Perkin Elmer 204-S fluorescent detector	WISP 710B and Integrator
7	Beckman 110B	Hitachi 1000 fluorescent detector	WISP 710B and Integrator
8	Rainin HPLX	Shimadzu RF-535 fluorescent detector	
9	Rainin HPLX	Shimadzu RF-535 fluorescent detector	

samples of the drug in biological samples. Other regression methods, including weighted linear regression, have been investigated for use in selected assays. The parameters of the standard curve are used to convert the peak height ratio of the drug peak to the internal standard peak in a chromatogram to the drug concentration of the clinical specimen.

Ten methodologies for 14 drugs and metabolites were under development or reports were in preparation or revision during the fourth year of the contract. Analytical methodologies that were at various stages of completeness during the past year in this laboratory are for the determination of the concentrations of the anti-leishmanial compound, WR 6026 in blood and plasma; the radioprotective drugs WR 2721 and WR 3689 in plasma; the anti-malarial compound WR 238,605 in blood and plasma; the anti-malarial drug, mefloquine in plasma; the anti-viral drug, ribavirin, and WR 249,992 in plasma; the anti-malarial drug, ß-arteether, and dihydroginghaosu in biological fluids, the anti-malarial drug, halofantrine, and its metabolite, WR 178,460, in blood and plasma; the 4-hydroxymethyl and mono desethyl metabolites of WR 6026 (WR 254,421 and WR 211,789) in biological fluids; and the carboxyl metabolite of mefloquine, WR 160,972, in plasma.

Routine assays for eight studies were underway during the fourth year of the contract. Routine analysis of 571 blood samples from the protocol titled "Multiple-Dose Pharmacokinetics, Safety and Tolerance of WR 6026 Hydrochloride in Healthy

Subjects" was reported in Analysis Report Wr6/B 88-7. Routine analysis of 62 plasma and 62 blood samples for the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methemoglobin Pharmacodynamics in the Beagle Dog" was reported in Analysis Report No. Wr5/BP 89-1. Routine analysis of 374 plasma samples for the protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of Pyridostigmine Administered by an Osmotic-Delivery Module (Osmet^{r}) compared to Pyridostigmine Syrup in Healthy Men" was reportedin Analysis Report No. Pyr/P 89-2. Routine analysis of 120 plasma samples for the protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of a Commercial Formulation of Sustained-Release Pyridostigmine in Healthy Men" was reported in Analysis Report No. Pyr/P 89-3. Routine analysis of 88 plasma and 88 blood samples for the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog" was reported in Analysis Report No. Pyr/P 89-4. Routine analysis of 240 plasma and 240 blood samples for the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog" was reported in Analysis Report No. Pyr/P 89-5. Routine analysis of 92 rat plasma, 13 rat bile, and 7 tube binding samples from the Walter Reed Army Institute of Research was reported in Analysis Report No. Pyr/P 89-6. Routine analysis of approximately 470 plasma and 470 blood samples for the World Health Organization was initiated and results will be submitted in Analysis Report No. Pyr/P 89-7.

DOCUMENTATION

Submittals Related to Contract DAMD17-86-C-6150 for 1989-90

Annual Reports

No. 3: covering 1988-9, dated Apr 17, 1989
No. 3, revised, submitted May 30, 1989

Project Status Reports

No. 13, dated July 12, 1989
No. 14, dated Oct. 2, 1989
No. 15, dated Jan. 2, 1990
No. 16, dated April 2, 1990

Study Reports

No. 10B: WR 6026 in plasma and blood, dated April 7, 1989
No. 10C: WR 6026 in plasma and blood, dated Sept. 14, 1989
No. 11B: WR 2721 in plasma, dated Sept. 28, 1989
No. 12B: WR 3689 in plasma, dated Nov. 14, 1989
No. 13A: WR 238,605 in plasma and blood, dated Mar. 29, 1989
No. 13B: WR 238,605 in plasma and blood, dated Nov. 17, 1989
No. 14B: Mefloquine in plasma, dated August 29, 1989

Submittals Related to Contract DAMD17-86-C-6150 for 1989-90 (Continued)

Analysis Reports

WR6/B 88-7: WR 6026 in blood, dated April 21, 1989
Mef/P 88-11B: Mefloquine in plasma, dated Jan. 31,1990
WR5/BP 89-1: WR 238,605 in beagle plasma and blood, dated April 13, 1989
Pyr/P 89-2A: Pyridostigmine in plasma, dated May 12, 1989
Pyr/P 89-2B: Pyridostigmine in plasma, dated November 28, 1989
Pyr/P 89-3A: Pyridostigmine in plasma, dated May 16, 1989
Pyr/P 89-3B: Pyridostigmine in plasma, dated November 30, 1989
WR5/BP 89-4A: WR 238,605 in beagle plasma and blood, dated June 1, 1989
WR5/BP 89-4B: WR 238,605 in beagle plasma and blood, dated Dec. 5, 1989
WR5/BP 89-5: WR 238,605 in beagle plasma and blood, dated Aug. 25, 1989
Phy/rP 89-6A: Physostigmine in rat plasma, dated Sept. 26, 1989
Phy/rP 89-6B: Physostigmine in rat plasma, dated Jan. 18, 1990

Publications

Published papers

High Performance Liquid Chromatographic Method for Detection of Physostigmine and Eseroline in Plasma using a Silica Gel Column and a Perchloric Acid Mobile Phase, *Journal of Liquid Chromatography*, **13(2)**,275-290(1990).

Planned papers

Ion-paired Liquid Chromatographic Method for the Analysis of Urine for Pyridostigmine.

Quantitation of WR6026 by HPLC.

STATUS OF ACCOMPLISHMENTS

The following review summarizes progress made on each of the methodologies under development and describes the routine analyses initiated during the fourth year of the contract.

WR 6026

In response to letters from the Contracting Officer (CO) dated March 10, 1989 and May 3, 1989, revisions to Study Report No. 10 "Quantitation of WR 6026 in Plasma and Blood by HPLC" were made and the report was resubmitted (draft B) on April 7, 1989 and (draft C) September 14, 1989.

Samples obtained according to the protocol titled "Multiple-Dose Pharmacokinetics, Safety and Tolerance of WR 6026 Hydrochloride in Healthy

Subjects" have been analyzed and Analysis Report Wr6/B 88-7 was completed on April 21, 1989.

WR 2721 AND WR 3689

Revisions dated September 28 and November 14, 1989, respectively, of Study Report No. 11 (draft B) "Quantitation of WR 2721 in Plasma by HPLC with Electrochemical Detection" and Study Report No. 12 (draft B) "Quantitation of WR 3689 in Plasma by HPLC with Electrochemical Detection" were made according to the CO's letter dated March 10, 1989.

WR 238,605

Development of an HPLC assay for WR 238,605 in plasma was completed, Study Report No. 13 "Quantitation of WR 238,605 as Free Base in Plasma and Blood by High Performance Liquid Chromatography and Fluorescence Detectio..' was submitted (draft A) March 13, 1989, and a revised Study Report 13 (draft B) in accordance with the CO's letter dated May 3, 1989 was submitted November 17, 1989. The quantitation concentration ranges were 0.815 - 408 ng/ml in plasma and 1.91 - 383 ng/ml in blood. The mobile phase was CH_3CN/water (1:1) and 5 mM $(NH_4)_2HPO_4$ (final concentration) adjusted to pH 7. Detection was by fluorescence with $\lambda_{emission}$ = 480 nm and $\lambda_{excitation}$ = 375 nm. Sample cleanup was by extraction with methyl *t*-butyl ether. Stability data has been obtained for plasma and blood samples stored at -20°C. Results indicate blood was not sufficiently stable for two month storage at this temperature. Additional data is being obtained to determine WR 238,605 stability in blood samples stored at -80°C and will be submitted at a future date.

Routine analysis of samples obtained according to the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methemoglobin Pharmacodynamics in the Beagle Dog" has been completed and Analysis Report Wr5/BP 89-1 was submitted April 13, 1989.

Routine analysis of samples obtained according to the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methemoglobin Pharmacodynamics in the Beagle Dog" has been completed and Analysis Report Wr5/BP 89-4 was submitted (draft A) June 1, 1989. Revisions to Analysis Report Wr5/BP 89-4 were made according to the CO's letter dated August 8, 1989 and the report was resubmitted (draft B) on December 5, 1989.

Routine analysis of samples obtained according to the protocol titled "Simultaneous Modeling of WR 238,605 Succinate Pharmacokinetics and Methemoglobin Pharmacodynamics in the Beagle Dog" has been completed and Analysis Report Wr5/BP 89-5 was submitted August 25, 1989.

MEFLOQUINE

Study Report No. 14 "Quantitation of Mefloquine (Free Base) in Plasma by High Performance Liquid Chromatography, Extraction Method" was revised according to the CO's letter of May 3, 1989 and was resubmitted (draft B) on August 29, 1989.

A revised version of Analysis Report No. Mef/P 88-11 (the routine analysis of plasma samples from Thailand for mefloquine free base concentrations) that makes

reference to the newer Study Report No. 14 was resubmitted (draft B) on January 31, 1990.

RIBAVIRIN

Study Report 15, "Quantitation of Ribavirin (WR 241,627) and AVS 206 (WR 249,992) in Plasma by HPLC," is in preparation.

In order to rapidly develop a method able to determine concentrations of both ribavirin (WR 241,627) and AVS 206 (WR 249,992), we concentrated our efforts on modification of the method of Granich, *et al* , in which ribavirin in plasma samples is initially separated from interfering substances on an affinity gel prior to separation by HPLC on a C18 silica gel column. Samples are analyzed twice, once with Granich's mobile phase to detect ribavirin and again with Granich's mobile phase modified by addition of butane sulfonic acid sodium salt to 0.06% to detect AVS 206. The ribavirin standard curve ranges 20 to 1000 ng/ml, and the AVS 206 standard curve ranges 10 to 1000 ng/ml.

Assay development is now focused on set up of a gradient mobile phase to reduce the run time from 30 min per sample. The first attempted gradient will flush the column with a 5% CH_3CN mobile phase immediately following elution of ribavirin and internal standard.

ß-ARTEETHER AND DIHYDROGINGHAOSU

Current tests for development of assays for WR 255,131 (ß arteether) and dihydroqinghaosu involve electrochemical detection, the same mobile phase (with the addition of 0.1% H_3PO_4, 10% CH_3CN and 0.5% H_2O_2) and the same phenyl column described in *J. of Chrom.* 414(1987)77-90 and UV photoirradiation. This system detects ß arteether at 10 ng/ml and dihydroqinghaosu at 50 ng/ml. However, the electrochemical detector becomes oxidized after only 200 samples.

Consideration is being given to derivitization of dihydroginghaosu (DQHS) and arteether to introduce a chromophore or to use fluorescence detection as an alternative approach to detection of dihydroqinghaosu and ß-arteether.

HALOFANTRINE

Improvements to the halofantrine assay methodology will be presented in Study Report No. 17, which will contain validation results for halofantrine in blood and plasma. Blood and plasma samples received October 5, 1989 have been analyzed. Analysis results will be reported in Analysis Report Hal/BP 89-7. Both reports are in preparation.

4-HYDROXY WR 6026

Preliminary data has been obtained for mobile phase constituents. The next samples to be run will test retention at lower pH's for the 40% CH_3CN mobile phase and in 20%, 70%, and 80% CH_3CN mobile phases at pH 7.

WR 160,972

We are testing C8 columns from Beckman, Whatman, and Alltech for separation of WR 160,972 (the mefloquine metabolite) from mefloquine. The analyst has requested another sample of mefloquine (WR 142,490) for development of this assay.

PYRIDOSTIGMINE

Routine analysis of plasma samples for determination of pyridostigmine concentrations for two studies began February 24, 1989. Analysis Report Pyr/P 89-2 (draft A) for samples obtained according to the protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of Pyridostigmine Administered by an Osmotic-Delivery Module (Osmetr) compared to Pyridostigmine Syrup in Healthy Men" was submitted May 12, 1989. A report revised according to the CO's letter dated August 8, 1989 was submitted (draft B) November 28, 1989.

Analysis Report Pyr/P 89-3 (draft A) for samples obtained according to the protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of a Commercial Formulation of Sustained-Release Pyridostigmine in Healthy Men" was submitted May 16, 1989. A report revised according to the CO's letter dated August 8, 1989 was submitted (draft B) November 30, 1989.

PHYSOSTIGMINE

Analysis Report No. Phy/rP 89-6 (draft A) "Routine Analysis of Physostigmine (Free Base) and Eseroline (Free Base) Rat Plasma, Bile and Tube Binding Samples Obtained from WRAIR" was submitted for review on September 26, 1989. A report revised according to the CO's letter dated October 31, 1989 was submitted (draft B) January 1, 1990.

TESTS

The following tests are conducted for the validation of a methodology under development. The sensitivity of the method is demonstrated by the analysis of prepared samples spiked at the drug concentration of the low point of the standard curve. The accuracy of the method is demonstrated by the analysis of blind samples provided by the US government. The reproducibility of the method is demonstrated by interday and intraday analysis of prepared replicate samples spiked at several concentrations. The recovery of the method is determined by comparison of the analyzed concentration of the drug spiked in a reference solution versus the analyzed concentration of the drug spiked in biological specimens. The stability of the drug in specimens is determined by analysis of samples that have been frozen for various lengths of time.